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Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA

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Multiple processing events are required to convert a single eukaryotic pre-ribosomal RNA (pre-rRNA) into mature 18S (small subunit), 5.8S and 25–28S (large subunit) rRNAs. We have asked whether U3 small nucleolar RNA is required for pre-rRNA processing *in vivo* by depleting *Saccharomyces cerevisiae* of U3 by conditional repression of U3 synthesis. The resulting pattern of accumulation and depletion of specific pre-rRNAs indicates that U3 is required for multiple events leading to the maturation of 18S rRNA. These include an initial cleavage within the 5′ external transcribed spacer, resembling the U3 dependent initial processing event of mammalian pre-rRNA. Formation of large subunit rRNAs is unaffected by U3 depletion. The similarity between the effects of U3 depletion and depletion of U14 small nucleolar RNA and the nucleolar protein fibrillarin (NOP1) suggests that these could be components of a single highly conserved processing complex.

Key words: RNA processing/ribosome synthesis/ *Saccharomyces cerevisiae*

Introduction

Eukaryotic cytoplasmic ribosomes consist of a small subunit containing 17–18S ribosomal RNA (rRNA) and a large subunit containing 5S, 5.8S and 25–28S rRNAs. Ribosome synthesis is initiated in the nucleolus where RNA polymerase I transcribes a single precursor rRNA (pre-rRNA) containing the domains destined to become the mature 17–18S, 5.8S and 25–28S rRNAs separated by internal transcribed spacers (ITS1 and ITS2) and flanked by 5′ and 3′ external transcribed spacers (ETSs). Rapid endonucleolytic cleavages of the pre-rRNA, which in mammals include an initial cleavage within the 5′ ETS, result in separation of the small and large subunit precursor components, which are subsequently matured as separate ribonucleoprotein (RNP) complexes (reviewed by Hadjiolov, 1985; Sommerville, 1986; Gerbi *et al.*, 1990; Reeder, 1990; and for yeast by Planta and Raué, 1988; Warner, 1989). Control of the early processing of pre-rRNA is possibly a key stage at which regulation of ribosome synthesis takes place. But are these processing events universally conserved, and how are they integrated with ribosome assembly?

This study focuses on the role of the U3 small nucleolar RNP (snoRNP) in these events. In mice and humans, the initial 5′ ETS cleavage event is dependent on U3 snoRNP *in vitro* (Kass *et al.*, 1990), and takes place immediately upstream of a nucleotide sequence that is conserved among vertebrates (Kass *et al.*, 1987; Bourbon *et al.*, 1988). U3 appears to be ubiquitous among eukaryotes (Gerbi *et al.*, 1990) and its function is essential in yeast (Hughes *et al.*, 1987). However, cleavage within the 5′ ETS has not been observed in yeast. Moreover, in *Xenopus* oocytes, U3 has been shown to be required for a different stage of pre-rRNA processing involving separation of the small and large subunit pre-rRNAs (Savino and Gerbi, 1990). Other models for U3 function have also been proposed, based on biochemical associations or nucleotide sequence complementarity between U3 and pre-rRNA (Prestayko *et al.*, 1970; Bachellerie *et al.*, 1983; Crouch *et al.*, 1983; Tague and Gerbi, 1984; Parker and Steitz, 1987; Kupriyanova and Timofeeva, 1988; Parker *et al.*, 1988), and cannot be completely excluded.

The 5′ ETS cleavage event can be reproduced using a mouse cell extract and synthetic RNA substrate. Efficient cleavage is dependent on conserved portions of the 5′ ETS within 300 nucleotides (nt) downstream of the cleavage site (Craig *et al.*, 1987, 1991). Direct evidence that U3 is required for this event was obtained by oligodeoxynucleotide-directed ablation of U3 by RNase H: cell extracts depleted of U3 in this way were unable to cleave the substrate (Kass *et al.*, 1990). The mouse cell extract supported accurate cleavage of a human substrate, indicating that the structural features of the RNA that determine the cleavage site are conserved and compatible among mammals (Kass *et al.*, 1987).

U3 and other snoRNPs have in common the properties of being precipitable with antibodies against the highly conserved nucleolar protein fibrillarin (Lischwe *et al.*, 1985; Hughes *et al.*, 1987; Parker and Steitz, 1987; Schimmang *et al.*, 1989; Tyc and Steitz, 1989; Tollervey *et al.*, 1991) and of copurifying with pre-rRNA upon cell fractionation (Prestayko *et al.*, 1970; Epstein *et al.*, 1984; Tollervey, 1987; Zagorski *et al.*, 1988; Tyc and Steitz, 1989). In yeast, two snoRNPs and fibrillarin have been shown to be important for pre-rRNA processing: when either U14 snoRNA or fibrillarin was depleted by conditional transcriptional repression, the pattern of accumulation and depletion of specific pre-rRNAs indicated that processing events necessary for generation of 18S rRNA and its precursors were inhibited (Li *et al.*, 1990; Tollervey *et al.*, 1991). These effects were accompanied by inhibition of growth and, in the case of fibrillarin depletion, by inhibition of methylation of pre-rRNA. The same processing events were found to be impaired in a cold-sensitive yeast strain carrying a deletion of the gene for the snoRNA, snR10 (Tollervey, 1987).

We have investigated the function of U3 in yeast by studying changes in the pre-rRNA processing pattern caused by repression of U3 synthesis. Our results show that in *Saccharomyces cerevisiae*, as in mammals, pre-rRNA is cleaved initially within the 5′ ETS, and that this cleavage event is U3 dependent. Furthermore, the cleavage events

that rapidly follow, generating the precursor to 18S rRNA, are also U3 dependent. The effects of U3 depletion, including inhibition of the initial 5′ ETS cleavage event, resemble the effects on processing observed when U14, snR10 and fibrillarin are depleted, suggesting that these four components may function as parts of a single processing complex.

Results

Depletion of U3 snoRNA by transcriptional repression

A yeast strain was constructed in which U3 synthesis could be repressed conditionally. U3 is encoded by two genes in haploid yeast (Hughes *et al.*, 1987). We replaced the wild-type gene for U3A with an allele which had been fused to a galactose upstream activator sequence, making its expression dependent upon galactose, in a strain in which the U3B gene had been disrupted, and then screened for galactose-dependent growth. As a control to distinguish specific defects due to depletion of U3 from non-specific defects accompanying inhibition of growth, a strain in which synthesis of U2 small nuclear RNA, required for pre-mRNA splicing, was dependent upon galactose was constructed in a similar way. The structure of the DNA fragments containing the conditional U3A and U2 alleles used to replace the cognate wild-type genes by homologous recombination is illustrated in Figure 1.

Both strains grew normally compared with isogenic control strains in liquid galactose medium (Figure 2A). When transferred to glucose medium, the growth rates remained normal for the first 6 h, with a doubling time of ~2 h, but then progressively decreased (Figure 2B). Whereas the repressible U2 strain ceased growth completely 22 h after transfer, the repressible U3 strain continued to grow, but progressively more slowly, attaining a 30 h doubling time after 55 h (Figure 2B). This indicated that either U3 was not absolutely required for growth in liquid medium, or residual synthesis of U3 was sufficient to support growth. (It was not possible to study the effects of repression for longer than 72 h owing to the selection for derepressed mutants.)

The level of U3 in the repressible U3 strain decreased after transfer to glucose (Figure 3A, lanes 1–6), falling successively to 26, 13, 6 and 3% of the initial level after 3, 6, 12 and 22 h, respectively. The level of U3 had fallen to 13% of the induced level, therefore, before a reduction in growth rate was observed. The rate of U3 depletion must be influenced by the rates of residual synthesis, of degradation and of dilution in the growing culture. Over the first 3 h, U3 was depleted with a half-life of ~1.5 h, which is consistent with the expected rate of depletion of a stable RNA in a culture with a doubling time of 2.1 h. After 3 h, the rate of U3 depletion decreased and the apparent half-life exceeded the doubling time of the culture, indicating that some residual transcription was occurring. We conclude, therefore, that transcriptional repression is effective in depleting U3, but that residual transcription could account for the continuing slow growth of the culture.

U3 in *S.cerevisiae* is spliced by the nuclear splicing apparatus (Myslinski *et al.*, 1990); however, the level of U3 in the repressible U2 strain after 22 h (Figure 3A, lane 8) remained approximately the same as in the control strains despite the accumulation of unspliced pre-U3 (Figure 3A,

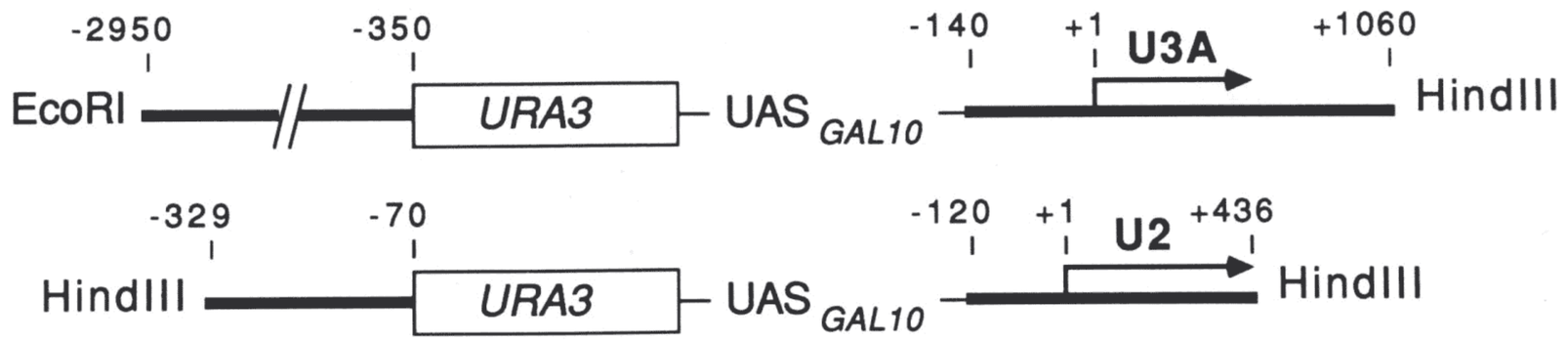

Fig. 1. Structure of DNA fragments used to replace the chromosomal U3A and U2 genes. The galactose-dependent U3A gene was substituted for the wild-type gene in a strain carrying a disruption of the U3B gene. Thick black lines represent sequences flanking the U3A (*SNR17A*) and U2 (*SNR20*) genes. Numbering is relative to the U3A and U2 transcription initiation sites. *URA3* is a yeast selectable marker; UAS_{GAL10} is a galactose upstram activator sequence.

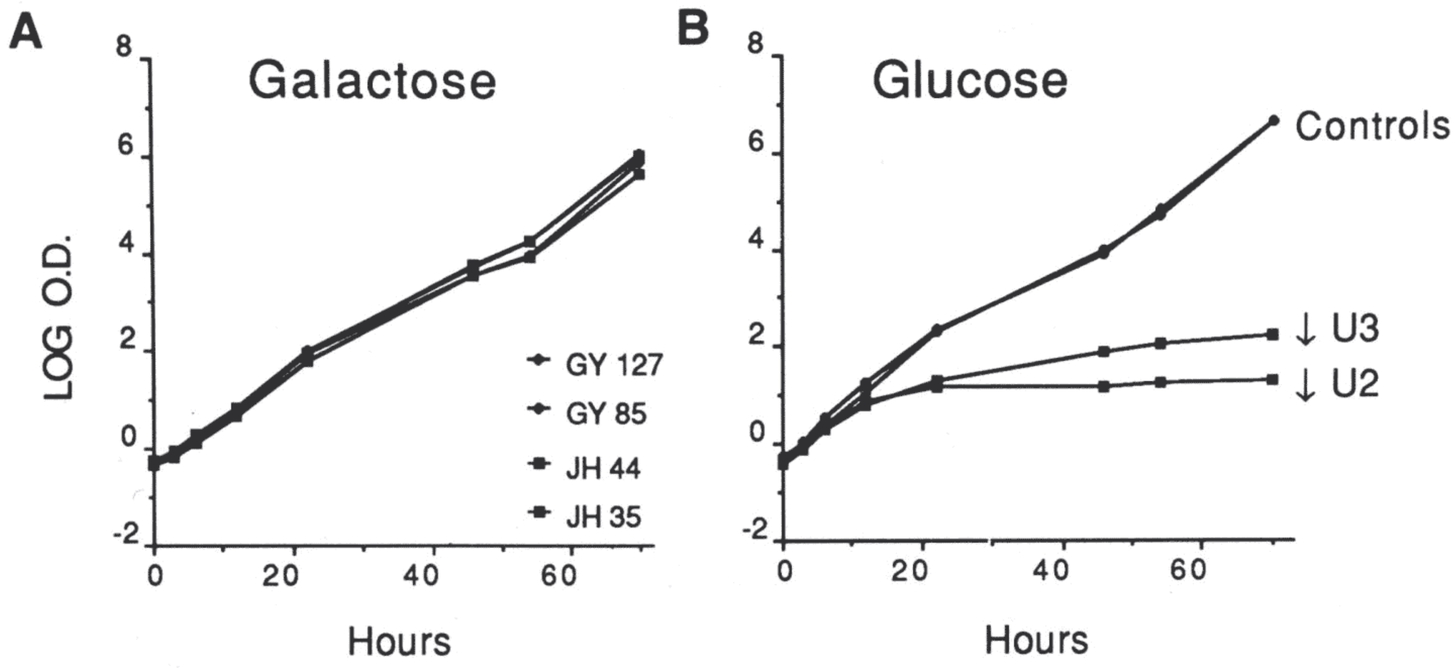

Fig. 2. Growth of repressible U3 and repressible U2 yeast strains in galactose (A) and glucose (B). Exponentially growing cultures in galactose medium were transferred at time '0' into fresh galactose or glucose medium. The cultures were diluted as necessary to maintain exponential growth. JH44 is the repressible U3 strain; GY127 is its isogenic control; JH35 is the repressible U2 strain; GY85 is its isogenic control. Cell density is expressed as LOG_{10} optical density at 600 nm.

lanes 7 and 9), indicating that the arrest of growth of this strain was not due to depletion of mature U3 following inhibition of splicing.

U3 is required specifically for formation of 18S rRNA

The most striking effect of U3 depletion was a decrease in the level of 18S rRNA relative to 25S rRNA concomitant with the decrease in growth rate (Figure 3B, lanes 1–6; compare Figure 2B). The 18S:25S ratio remained unchanged for up to 6 h after the transfer but then declined abruptly (Figure 3C). The levels of other RNA species relative to 25S rRNA, such as tRNAs, 5S and 5.8S rRNAs, scR1 (Felici *et al.*, 1989) and snR30 (Bally *et al.*, 1988), detected by ethidium bromide staining in denaturing polyacrylamide gels, remained normal (data not shown). The 18S:25S ratios in the repressible U2 strain (Figure 3B, lane 8) and in the control strains (lanes 7 and 9) after 22 h were normal. These results indicate that U3 depletion specifically inhibits accumulation of 18S rRNA, without obviously affecting 5.8S or 25S rRNA, despite the fact that these RNAs are derived from the same 35S pre-rRNA. The specific depletion of 18S rRNA could have been caused by destabilization of small subunit pre-rRNA components, or inhibition of specific processing events required for 18S rRNA formation.

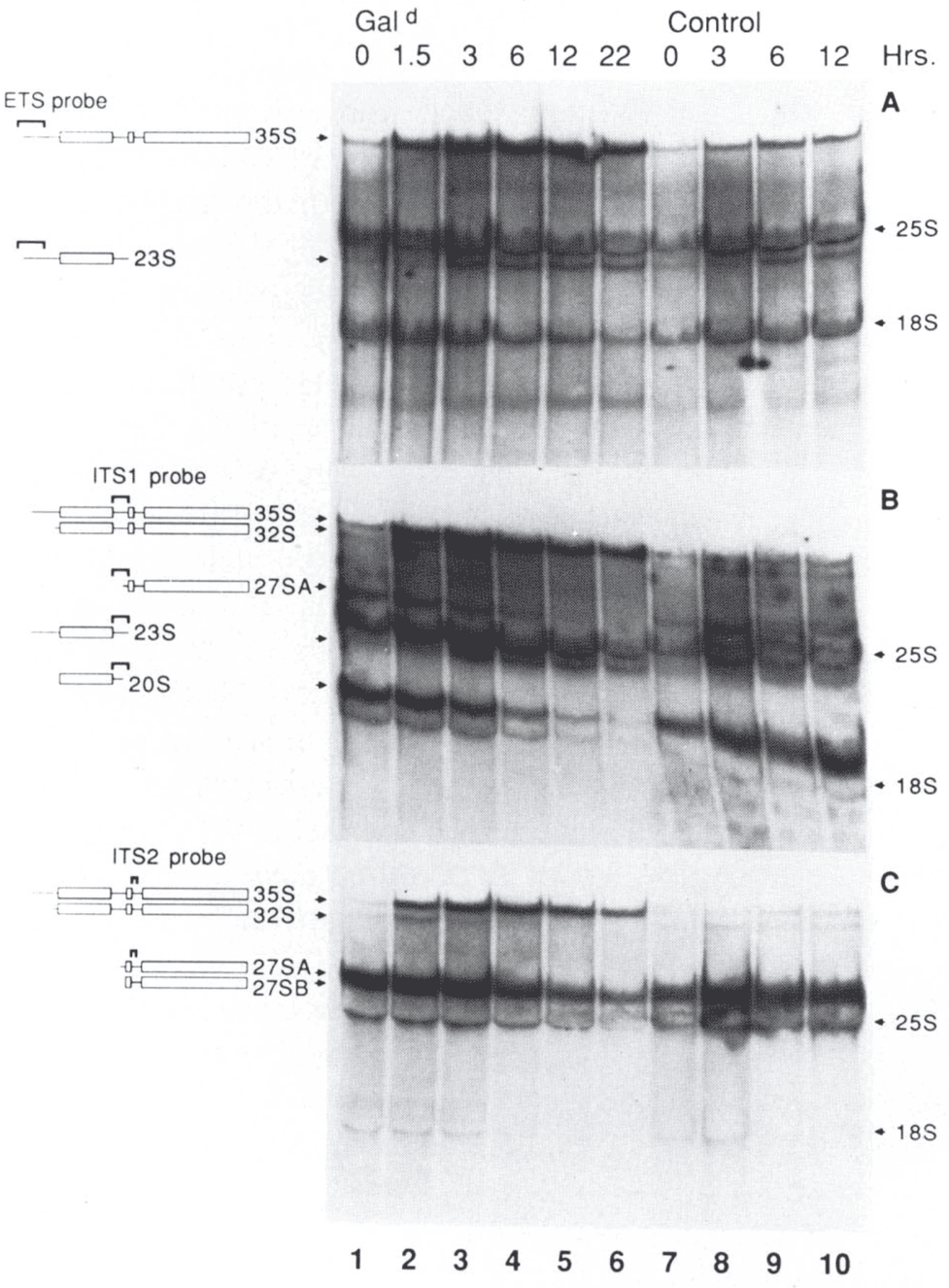

Fig. 3. Effects of U3 depletion on the levels of 18S and 25S rRNAs. Total RNA was extracted at the indicated times after the transfer to glucose medium of the repressible U3 strain (lanes 1–6), the repressible U2 strain (lane 8), and the two isogenic control strains (lanes 7 and 9), and analyzed by **A**: Northern blotting, with a probe for U3A (the lower band in lane 8 is a stable product of pre-U3 degradation), **B**: agarose gel electrophoresis and ethidium bromide staining, and **C**: densitometric scanning of photographic negatives of ethidium bromide stained gels. Equal quantities of RNA were loaded in each lane.

U3 depletion inhibits early processing events that generate the small subunit pre-rRNA

The yeast 18S, 5.8S and 25S rRNAs are derived from a 35S pre-rRNA that extends from 699 nt upstream of the 5′ end of the 18S domain (Bayev *et al.*, 1980; Klemenz and Geiduschek, 1980) to 7 nt downstream of the 3′ end of the 25S domain (Veldman *et al.*, 1980b; Yip and Holland, 1989); this is the longest pre-rRNA that forms a detectable pool, but it does not represent the full-length product of transcription, which appears to terminate 210 nt downstream of the 3′ end of the 25S domain (Kempers-Veenstra *et al.*, 1986). The 35S pre-rRNA is rapidly processed by removal of at least 0.5 kb of the 5′ ETS, generating a transient 32S RNA (Klemenz and Geiduschek, 1980; Tollervey, 1987; Veinot-Drebot *et al.*, 1988), which is then cleaved within ITS1 to give two relatively stable intermediate RNAs: the 20S small subunit and the 27SA large subunit pre-rRNAs (Veldman *et al.*, 1980a). The 5′ end of the 20S RNA corresponds to the mature 5′ end of 18S rRNA (De Jonge *et al.*, 1977). Conversion of 20S RNA to 18S rRNA by removal of the remaining 209 nt of ITS1 from the 3′ end occurs after the small subunit precursor has been exported to the cytoplasm (Udem and Warner, 1972; Trapman and Planta, 1976). 27SA RNA is converted to 27SB by removal of most or all of the remaining 146 nt of ITS1 from the 5′ end (which is left heterogeneous; Rubin, 1974), and the remaining 7 nt from the 3′ end. The 27SB RNA is

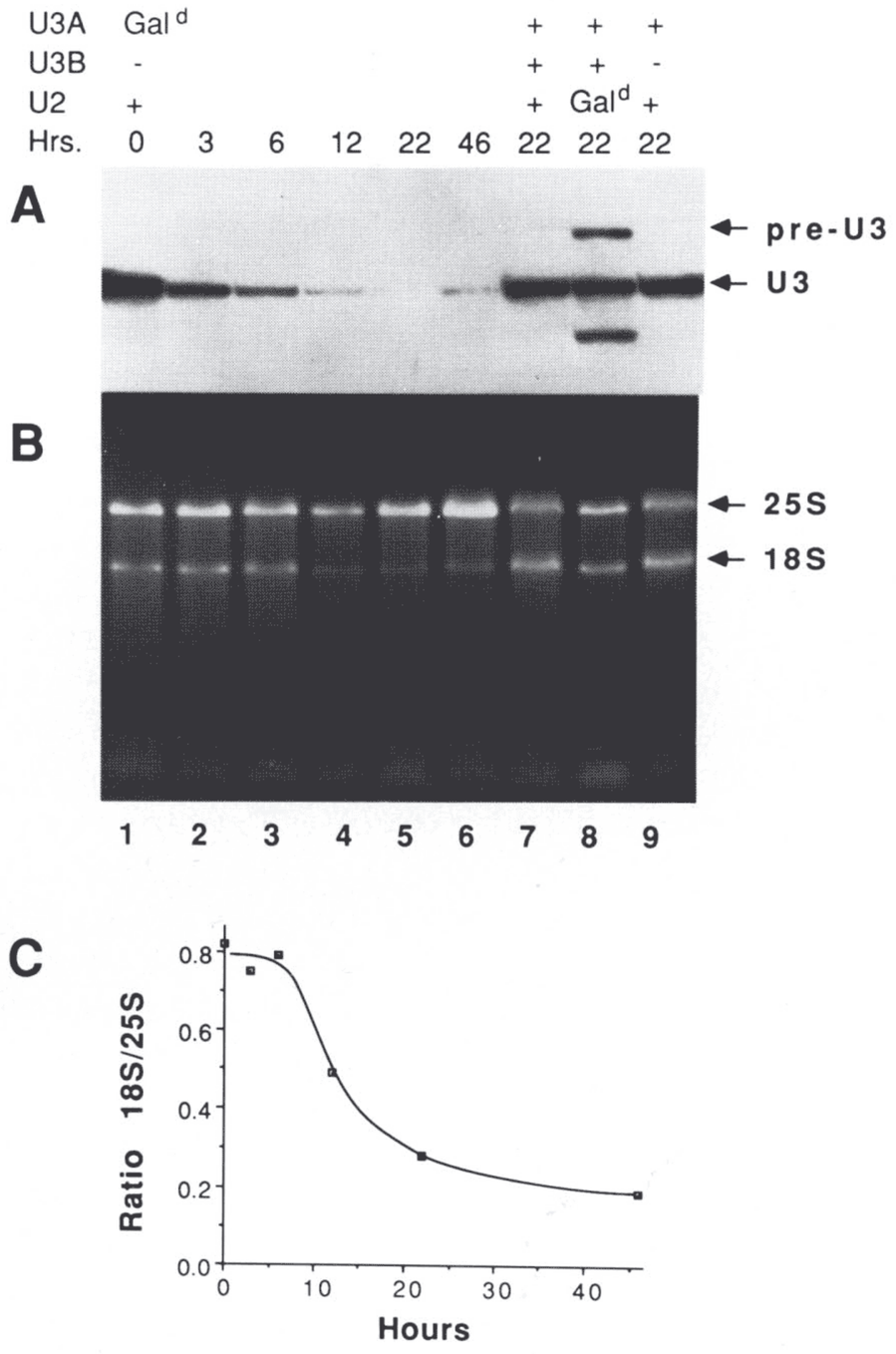

Fig. 4. Effects of U3 depletion on the levels of pre-rRNA. Northern blots of total yeast RNA extracted at the indicated times after the transfer to glucose of the repressible U3 strain (lanes 1–6), and the isogenic control strain (lanes 7–10). The probes were complementary to the 5′ 200 nucleotides of the 5′ ETS (**A**); the complete ITS1 (**B**); and the 5′ 194 nucleotides of ITS2 (**C**). The 18S and 25S rRNAs are visible as shadows.

subsequently converted to the mature 5.8S and 25S rRNAs by a multi-step process which removes ITS2 (Veldman *et al.*, 1981).

The cleavage event that forms the 5′ end of the 20S RNA has been referred to as 'A_1'; that which forms the 3′ end of the 20S RNA and the 5′ end of the 27SA RNA, as 'A_2'; and that which forms the 5′ end of 27SB RNA, as 'B_1' (Veldman *et al.*, 1981; see Figure 7A).

To determine at which stage in the processing pathway formation of 18S rRNA was blocked, the levels of the various pre-rRNAs were analyzed by hybridization on Northern blots using three pre-rRNA-specific probes, complementary to the 5′ portion of the 5′ ETS, to the complete ITS1, and to most of the ITS2 (see Materials and methods).

The earliest detectable effect of U3 depletion was accumulation of the 35S pre-rRNA. No change in the level of 35S RNA was observed 45 min after the transfer to glucose (data not shown), but accumulation was clearly apparent after 1.5 h (Figure 4A, B and C, lanes 1 and 2), and reached a maximum after 3 h (Figure 4A, B and C, lane 3), at which time, the level was 4- to 5-fold higher than the initial value; the growth rate at this time was still normal (Figure 2B), but U3 had been substantially depleted (Figure 3A, lane 2). The level of 35S RNA accumulation decreased after longer periods (Figure 4A, B and C, lanes 4−6), probably reflecting secondary effects due to the decreased growth-rate.

The early accumulation of 35S pre-rRNA suggests that the primary effect of U3 depletion was inhibition of one or more initial cleavage events. Consistent with this, the cleavage products 32S, 27SA and 20S RNAs were depleted subsequent to the accumulation of 35S pre-rRNA (Figure 4B and C, lanes 1−6). Despite the depletion of 27SA RNA, however, the level of 27SB RNA was relatively unaffected, showing only a slight decline after longer periods (Figure 4C, lanes 1−6) in the same way that the level of 35S RNA declined after its initial accumulation. This indicates that 27SA RNA is not an obligatory precursor of 27SB RNA, and that 27SB can be derived directly from 35S pre-rRNA by cleavage B_1. In support of this, a 23S RNA, the expected 5′ B_1 cleavage product of 35S pre-rRNA, consisting of the 18S domain flanked by the 5′ ETS and ITS1, can be seen to accumulate (Figure 4A and B, lanes 1−3). 23S RNA was also observed to accumulate upon depleting yeast cells of U14 snoRNA and of fibrillarin (Li *et al.*, 1990; Tollervey *et al.*, 1991), and was observed at elevated levels in a strain carrying a deletion of the snR10 gene (Tollervey, 1987). 23S RNA appears to be a normal component of the processing pathway, as it is also detectable in the control RNA samples, although at lower levels (Figure 4A and B, lanes 1 and 7−10); the abundance of 23S RNA, therefore, appears to be determined by the rates of the cleavage steps which generate 32S, 20S and 27SA RNAs relative to the rate of B_1. No other intermediate processing products were detected.

We conclude that U3 depletion inhibits the processing of 35S pre-rRNA to 32S, 27SA and 20S RNAs, but does not affect cleavage B_1, which generates 27SB RNA. Formation of 20S and 27SA RNAs involves cleavages A_1 (at the 5′ ETS−18S boundary) and A_2 (within ITS1) (De Jonge *et al.*, 1977; Veldman *et al.*, 1980a). The hybridization pattern of 32S RNA indicates that it lacks at least 0.5 kb of the 0.7 kb 5′ ETS (Tollervey, 1987; Veinot-Drebot *et al.*, 1988), however, it is not known whether 32S RNA is generated by A_1, or by an even earlier cleavage event taking place within the 5′ ETS. Initial processing of mammalian pre-rRNA involves cleavage within the 5′ ETS at a site between 0.4 kb and 0.8 kb from the 5′ end (Craig *et al.*, 1987; Stroke and Weiner, 1989) and is U3 dependent *in vitro* (Kass *et al.*, 1990); we therefore sought to determine whether a similar processing event occurs in yeast.

U3 dependent processing event within the yeast 5′ ETS

A series of primer extension reactions were performed on the RNA samples from the experiment shown in Figure 4, using two different oligodeoxynucleotide primers. The first primer was complementary to the 3′ end of the 5′ ETS (nucleotides −29 to −1 relative to the ETS−18S boundary) and the second primer was complementary to 18S rRNA close to the 5′ end (nucleotides +30 to +57). The first primer generated two prominent extension products (Figure 5A, lanes 1−11, bands a and c) and the second primer generated three (lane 12, bands a, b and d). The longest products of both primers (Figure 5A, bands a) probably extend to the 5′ end of the ETS (nucelotide −699 with respect to the ETS−18S boundary), the second longest products (lanes 1−11, band c; lane 12, band b) extend as far as nucleotides −89 (major band) and −90 (minor band) of the ETS, and the third product of the second primer (lane 12, band d) extends to the 5′ end of the mature 18S rRNA (nucleotide +1). The intensity of the putative full-length extension product is greater after transfer of the repressible U3 strain to glucose (Figure 5A, lanes 2−6, band a) than after the transfer of the control strain (lanes 8−11, band a), consistent with the accumulation of the 35S and 23S RNAs observed upon U3 depletion by Northern blotting (Figure 4). The intensity of the '−89/−90' product decreases with time after transfer of the repressible U3 strain to glucose (Figure 5A, lanes 1−6, band c) but remains relatively constant in the samples from the control strain (lanes 7−11, band c). The same pattern of depletion of the '−89/−90' product was also observed using the second primer (data not shown). (The faint extension product in Figure 5A, lanes 1−11, similar in size to band b, does not map to the same position as the faint extension product in lane 12 above band b; we believe that these are artefacts due to ectopic priming.)

These results show that a U3 dependent processing event takes place within the 5′ ETS. It appears to represent the initial processing event within the 5′ ETS, as no other reverse transcriptase products were detected that were common to both primers, and it can precede cleavage at the ETS−18S boundary (A_1), since it could be detected by extension of a primer complementary to 18S rRNA (Figure 5A, lane 12). The processing event could be a cleavage or a nucleotide modification causing reverse transcriptase to terminate. The heterogeneity of the −89/−90 cDNA product could be due to heterogeneously cleaved 5′ ends, or to the addition of an extra, untemplated 3′ terminal nucleotide.

To distinguish between a cleavage event and a nucleotide modification, we analyzed this site further using a ribonuclease-protection assay, reasoning that most nucleotide modifications are unlikely to be detectable by this method.

A radioactively labelled RNA transcript complementary to the pre-rRNA from −181 to +62 (relative to the ETS−18S boundary) was hybridized to the RNA samples used in the previous experiment, digested with ribonuclease T1, which cleaves single-stranded RNA after G residues, and analyzed by electrophoresis. Three major digestion products were detected: the longest product (Figure 5B, lanes 4−13, band e) was approximately of the size predicted (254 nt) for protection by pre-rRNA with the full-length 5′ ETS; the shortest product (Figure 5B, lanes 4−13, band g) was approximately of the size predicted (82 nt) for protection by mature 18S rRNA; and the product of intermediate length (Figure 5B, lanes 4−13, band f) was approximately of the size predicted (159 nt) for protection by pre-rRNA with a 5′ end within the 5′ ETS at nucleotide −89, where the strong U3 dependent reverse transcriptase termination was observed. This product of intermediate length decreased in intensity after transfer to glucose of the repressible U3 strain (Figure 5B, lanes 4−9, band f), but not of the control strain (lanes 10−13, band f), and therefore confirms the existence of a U3 dependent processing event within the 5′ ETS. A similar band pattern was observed when ribonuclease A was used in the assay instead of ribonuclease T1 (data not shown). [A band of similar mobility to 'f', visible in the undigested control lanes (Figure 5, lanes 1 and 3), appears to be an artefact as it does not persist after RNase digestion (lanes 2 and 9). We cannot make conclusions based on quantitative differences in band intensities in this experiment (beyond noting the depletion of the '−89' product), owing to effects of competition for the probe which was not in molar excess.]

These results show that an early U3 dependent processing event takes place within the 5′ ETS of *S.cerevisiae* pre-rRNA. The fact that this event is detectable both by primer extension and by ribonuclease protection assays argues that it represents a cleavage event. The predominant cleavage appears to take place between nucleotides 609 and 610 from the 5′ end of the 5′ ETS, 89 nt upstream of the 5′ ETS−18S boundary. We refer to this cleavage event as 'A_0'.

Because A_0 can precede A_1, at least in a proportion of cases, it must generate the 5′ end of a proportion of 32S RNA. We have confirmed this by Northern blotting: we found that a probe complementary to nucleotides −87 to −1, relative to the 5′ ETS−18S boundary, hybridizes to 32S RNA (data not shown). The same probe did not

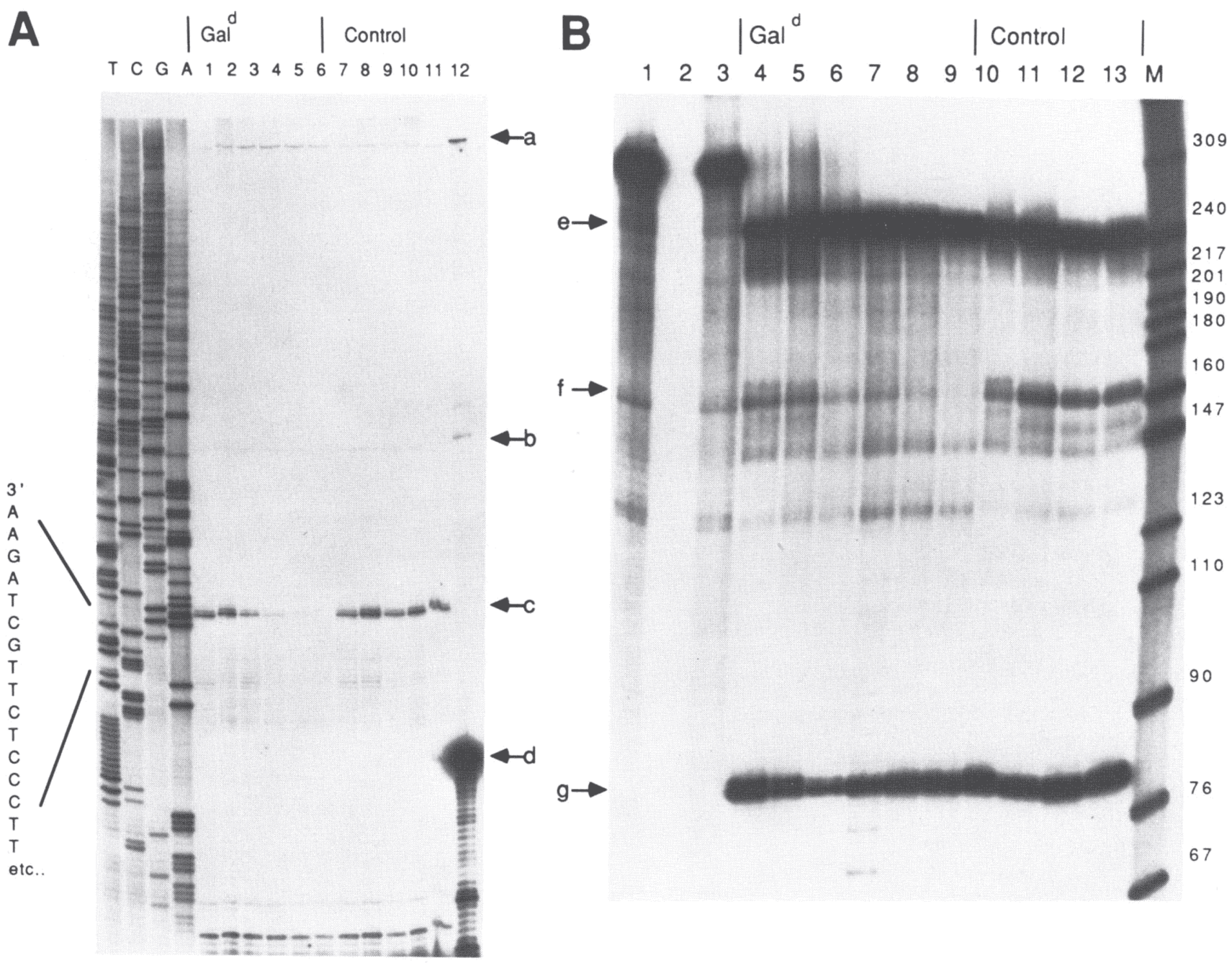

Fig. 5. Analysis of the yeast 5′ ETS processing site. **A**. Primer extension reactions were performed on RNA extracted at 0, 1.5, 3, 6, 12 and 22 h after transfer to glucose of the repressible U3 strain (lanes 1−6), or 0, 3, 6, 12 and 22 h after transfer of the isogenic control strain (lanes 7−11), using an oligonucleotide complementary to the 5′ ETS (nucleotides −29 to −1 relative to the ETS−18S boudary). The same oligonucleotide was used to generate the sequence ladder from a plasmid DNA template. A primer extension reaction using a second oligonucleotide, complementary to 18S rRNA (nucleotides +30 to +57), was performed on the 12 h control sample (lane 12). The major primer extension products correspond to the 5′ end of the ETS (a); nucleotide −89 (b in lane 12; c in lanes 1−11); and the 5′ end of 18S rRNA (d in lane 12). The 3′ end of the complementary DNA sequence at −89 is indicated. **B**. Ribonuclease protection analysis. A uniformly labelled probe, complementary to nucleotides −181 to +62 (relative to the ETS−18S boundary), was annealed to RNA extracted 0, 1.5, 3, 6, 12 and 22 h after transfer of the repressible U3 strain to glucose (lanes 4−9), or 0, 3, 6 and 12 h after transfer of the control strain (lanes 10−13), and then digested with RNase T1. As controls, the undigested probe alone (lane 1), the digested probe alone (lane 2) and the undigested probe annealed to RNA (lane 3) are shown. Prominent protected products are indicated (e, f and g).

hybridize to the 20S RNA, suggesting that A_2 does not precede A_1.

Comparison of the 5′ ETS processing sites of different organisms

Processing within the 5′ ETS has been observed in several eukaryotes, and may be a universal feature of pre-rRNA processing. However, 5′ ETS structures are extremely diverse, both in nucleotide sequence and in length, and the positions of processing sites within 5′ ETSs are variable, so it is not clear whether these processing events are homologous. Processing sites in human, mouse (Kass *et al.*, 1987), rat (Stroke and Weiner, 1988) and *Xenopus* (E.Mougey and B.Sollner-Webb, personal communication) occur immediately upstream of an identical sequence of 11 nt, the most conserved sequence within the vertebrate 5′ ETS; in the mouse RNA, this constitutes part of a 28 nt minimum substrate for cleavage *in vitro* (Craig *et al.*, 1991). We found little obvious resemblence between this sequence and the sequence downstream of the yeast A_0 site (or any other part of the yeast 5′ ETS), so we compared the sequences around other known 5′ ETS processing sites to look for more subtle but conserved similarities. The sequences in Figure 6 were first aligned according to the mapped 5′ ends of the processed products, the alignment was then adjusted slightly to show the best match to the conserved vertebrate sequence. [Brine shrimp (Koller *et al.*, 1987) does not appear to match this sequence, and has not been included in Figure 6.] The first striking common feature of these sequences is the presence (except in the silk moth) of a short stretch of pyrimidines at or close to the processing sites (boxed in Figure 6). Secondly, the recurrence of four G residues and an A at positions downstream of the cleavage sites (bold face in Figure 6) suggests a consensus of a few broadly conserved nucleotides. We suggest that these features might be significant elements in a common recognition site for components of a processing complex.

Discussion

We have studied the function of U3 snoRNA *in vivo* by analyzing the changes in the pre-rRNA processing pattern caused by repression of U3 synthesis. Our results yield three main conclusions. First, U3 depletion causes inhibition of multiple processing events that are necessary for the formation of 18S rRNA, but not 5.8S and 25S rRNAs. Second, we have identified a U3 dependent processing event in the yeast 5′ ETS analogous to that which occurs in mammals, indicating that mechanisms of pre-rRNA processing and U3 function have been conserved. Third, the effects of U3 depletion are similar to those caused by deficiencies in other highly conserved nucleolar components, suggesting that their functions are intimately related to that of U3.

U3 is required for multiple processing events in 18S rRNA formation

The earliest detectable effect of U3 depletion was accumulation of the 35S pre-rRNA followed by depletion of the intermediate 32S, 27SA and 20S RNAs (Figure 4). This effect was observed within 1.5 h of the onset of repression, at which time the level of U3 had been reduced by half, suggesting that U3 might have a direct role in these processing events. Depletion of 18S rRNA followed depletion of its immediate precursor, 20S RNA, and was first observed after 6 h (Figure 3), concomitant with the decline in growth rate (Figure 2). This indicates that the inhibition of growth was probably due to the reduced ability to generate small ribosomal subunits. The levels of 5.8S and 25S large subunit rRNAs did not appear to be affected directly by U3 depletion: the precursor of these molecules, 27SB RNA, continued to be generated after the accumulation of 35S pre-rRNA by a U3-independent cleavage event, B_1, which effectively separates the large and small subunit pre-rRNA processing pathways. That 35S pre-rRNA was cleaved directly at B_1, was indicated by the accumulation of the upstream product of this cleavage, 23S RNA (Figure 4).

The U3 dependent processing events include cleavage A_0, which we have identified by primer extension and ribonuclease protection assays, and takes place within the 5′ ETS, 89 nt upstream of the ETS−18S boundary (Figure 5), and cleavages A_1 and A_2, which take place at the 5′ ETS−18S boundary and within ITS1, respectively (Veldman *et al.*, 1980a). We assume the usual order of these events to be A_0, A_1, A_2, as primer extension analysis indicates that A_0 can precede A_1 (Figure 5), and the existence of 32S RNA indicates that A_0 and A_1 precede A_2; this order of cleavage, however, may vary in a fraction of

Fig. 6. Comparison of processing sites in the 5′ external transcribed spacers of pre-rRNAs. Possibly significant elements of a common recognition site for processing are indicated by bold face and boxes. Positions of the 5′ ends of processed products are indicated by arrows if pricisely mapped, or by ~ ~ ~ if approximately mapped, for human and mouse (Kass *et al.*, 1987), rat (Stroke and Weiner, 1989), silk moth (Fujiwara and Ishikawa, 1987), *Tetrahymena* (Sutiphong *et al.*, 1984), *Physarum* (Blum *et al.*, 1986) and *Neurospora* (Tyler and Giles, 1985). Processing has also been observed in *Xenopus* (E. Mougey and B. Sollner-Webb, personal communication). Only the yeast 5′ end corresponding to the major reverse transcriptase product (of the doublet at band c, Figure 5A) has been marked. Numbers relate to the 5′ ends of the pre-rRNAs.

cases. It would seem likely that the 5′ ends of the 32S RNA could be heterogeneous, formed by either A_0 by A_1.

We do not know how A_0, A_1 and A_2 are dependent on U3. Perhaps they have independent requirements for U3, or perhaps only A_0 requires U3 directly and A_1 and A_2 are constrained to occur only after A_0 has taken place. If the former is true, then the question arises why A_0 is necessary, and if the latter is true, then what is the mechanism of the constraint? Processing analogous to A_1 has been reproduced *in vitro* using a human nucleolar extract and a short synthetic human substrate, and was found to be insensitive to micrococcal nuclease treatment (Hannon *et al.*, 1989), suggesting that an RNA component may not be necessary for this processing event; but perhaps extra constraints would be imposed by a longer substrate. A cleavage event perhaps equivalent to A_2 has been observed *in vivo* within the mouse ITS1, and there was some suggestion that this might be dependent on the presence of an intact 5′ ETS on the pre-rRNA (Raziuddin *et al.*, 1989). It remains to be determined whether cleavage equivalent to A_2 occurs in the ITS1 of other organisms. Depletion of U3 from *Xenopus* oocytes impairs cleavage at, or close to the ITS1−5.8S boundary (Savino and Gerbi, 1990): this result would be consistent with our data if a cleavage event equivalent to A_2 takes place near the 3′ end of the *Xenopus* ITS1.

Our data lead to a revision of the yeast pre-rRNA processing pathway, as shown in Figure 7. The steady-state levels of precursor and intermediate RNAs are determined by the rates of the U3 dependent processing events, A_0, A_1 and A_2, relative to the U3 independent B_1 event. The rates of the 'A' cleavages are directly related to the level of U3, such that, under 'wild-type' conditions, the majority of the 35S pre-rRNA is rapidly cleaved at A_0, A_1 and A_2 (Figure 7A). Upon U3 depletion, the reduced 'A' cleavage rate leads to reduced levels of the 32S, 27SA and 20S RNAs, and accumulation of the 35S and 23S RNAs (Figure 7B). At low levels of U3, most of the 23S RNA must be degraded, as it is synthesized stoichiometrically with the large subunit pre-rRNA. In our experiments, incomplete repression of U3 synthesis could have supported the slow conversion of 23S RNA to 18S rRNA, accounting for the continued slow growth of the culture.

A conserved U3 dependent processing event in the 5′ ETS

Cleavage within the 5′ ETS seems to be a conserved feature of pre-rRNA processing. By aligning the 5′ ETS processing sites of several eukaryotes, we have observed weak sequence similiarities which may reflect common structural features recognized by components of the processing machinery. The discovery that the 5′ ETS processing events in mammals and yeast are dependent on U3 implies that the mechanism of cleavage and the function of U3 has been conserved. Psoralen cross-linking studies have shown that U3 is assocoated with the 5′ ETS in mammals (Stroke and Weiner, 1989; Maser and Calvet, 1989), and complexes containing both the substrate for *in vitro* processing and U3 have been detected by native gel electrophoresis (Kass *et al.*, 1990); however, we do not know how U3 is involved in the cleavage reaction. Using a mouse cell extract, specific cleavage of the 5′ ETS substrate is U3 dependent (Kass *et al.*, 1990), but it has also been shown that specific cleavage can be reproduced with a highly purified nucleolar ribonuclease which does not appear to be a snoRNP (Shumard and Eichler, 1988), suggesting that U3 could be required for the integrity and activity of a processing complex in cell extracts and *in vivo*, but that the component of the complex directly responsible for the endonucleolytic activity can function independently when purified.

U3 and other nucleolar components are required for the same early processing events

U3 in yeast appears to be required for the same processing events as the highly conserved nucleolar protein fibrillarin (Tollervey *et al.*, 1991) and the snoRNAs U14 (Li *et al.*, 1990) and snR10 (Tollervey, 1987): the effects of depletion *in vivo* of each of these components are consistent with inhibition of A_0, A_1 and A_2. How the functions of these components are related is not clear. Human fibrillarin functions in yeast but causes aberrations in nuclear morphology, suggesting a fundamental role in organizing nucleolar structure (Jansen *et al.*, 1991). It is not known how the nucleolus assembles around rRNA genes, but part of this process could involve recognition of specific sites on nascent pre-rRNA by components required for ribosome assembly and processing. It is puzzling why the 5′ ETS of eukaryotic,

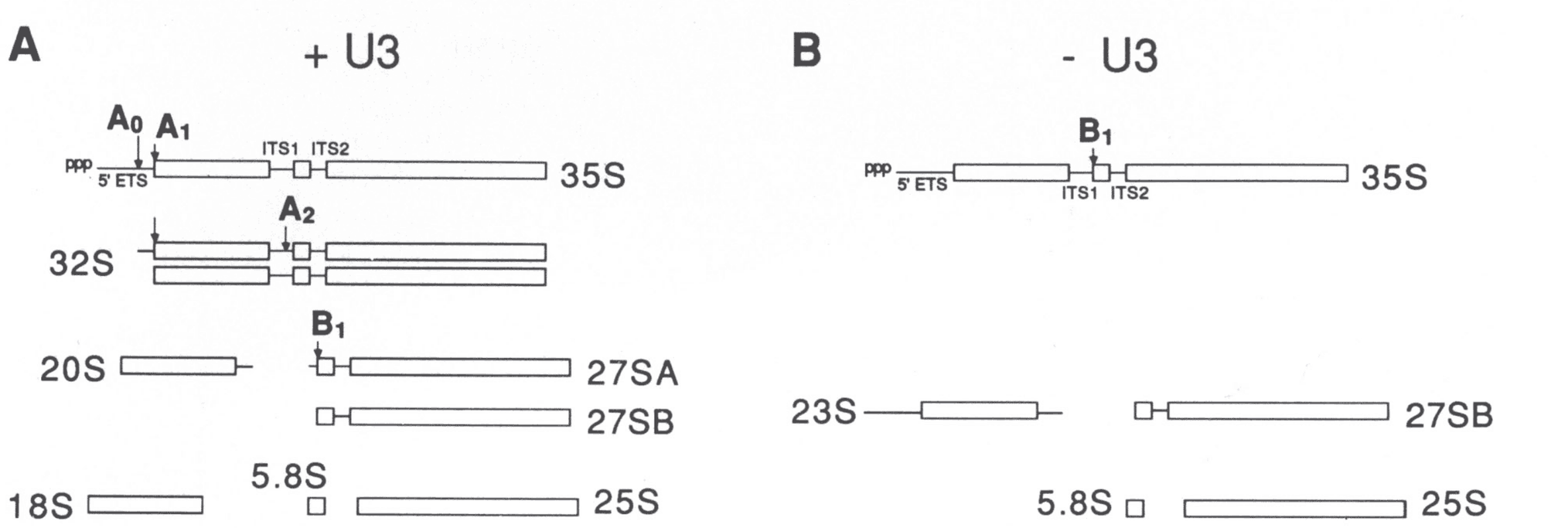

Fig. 7. Yeast pre-rRNA processing in the presence and absence of U3 snoRNA. **A**. Cleavage events A_0, A_1 and A_2 occur rapidly when the steady-state level of U3 is high, causing most 35S pre-rRNA to be converted to 32S, 27SA and 20S RNAs before cleavage event B_1 occurs. 32S RNA may have heterogeneous 5′ ends, formed by A_0 and A_1. **B**. At low levels of U3, A_0, A_1 and A_2 occur slowly, most 35S pre-rRNA is cleavaged first at B_1, and 35S and 23S RNAs accumulate. The levels of 27SB, 5.8S and 25S RNAs are not significantly perturbed by U3 depletion. Excess 23S RNA is presumably degraded.

particularly mammalian, pre-rRNA is so long; deletions within the yeast 5′ ETS and ITS1 abolish formation of 18S rRNA, indicating the importance of these spacers (Musters *et al.*, 1990). Perhaps U3, U14, snR10, and other snoRNPs in association with fibrillarin interact with the 5′ portion of pre-rRNA and directly mediate initial processing events, or enhance or prevent specific RNA folding relevant to the function of the ribosome; cleavages within the pre-rRNA spacers may then take place only with correctly assembled precursor, and serve to promote further assembly events.

Materials and methods

Plasmid and strain construction

A plasmid containing a yeast DNA fragment bearing a repressible U3 gene that could be used to replace the corresponding chromosomal locus was constructed as follows. The genomic 1.4 kb *Hin*dIII fragment containing *SNR17A* (Hughes *et al.*, 1987) was inserted into the *Hin*dIII site of the yeast expression vector pEMBLyex4 (Cesareni and Murray, 1987) in the downstream orientation with respect to the galactose upstream activator seqence (UAS_{GAL}). The UAS_{GAL} was then brought into closer proximity to the U3 gene promoter by deleting the 0.5 kb fragment between the *Xho*I site of pEMBLyex4 and the *Afl*II site at −140 with respect to the U3 transcription initiation site (defined by Myslinski *et al.*, 1990). A single *Stu*I−*Msc*I fragment containing the 3′ portion of *URA3*, the UAS_{GAL} and the entire *SNR17A* gene was substituted for the equivalent *Stu*I−*Msc*I fragment (lacking the UAS_{GAL}) of a plasmid containing the *snr17a*::*URA3* allele used in the U3 gene deletion analysis (Hughes *et al.*, 1987). The resultant plasmid pRexA10 contains a 5′ portion of *SNR17A* fused to the *URA3*:*snr17a*-Gal^d cassette on a single *Eco*RI−*Hin*dIII fragment (Figure 1), which could be used to replace the yeast chomosomal *SNR17A* allele. The plasmid pUraGalU2 that enables the replacement of the yeast chromosomal U2 gene with a repressible U2 gene was constructed in a similar way as described (Miraglia *et al.*, 1991).

Yeast strain JH44, which carries a disruption of the U3B gene and is dependent on galactose for expression of U3A, was derived by transforming GY127 (*MAT*a *snr17b*::*LEU2 ura3*-52 *his3*-Δ *trp1 lys1*-1 *ade2*-1 *can1*-100) with the *Eco*RI−*Hin*dIII fragment of pRexA10, selecting for uracil prototrophy and screening for galactose-dependent growth. JH35, which is galactose-dependent for U2 expresion, was derived similarly by transforming GY85 (*MAT*α *ura3*-52 *leu2*-3,12 *his3*-Δ *lys1*-1 *ade2*-1 *can1*-100) with a single *Hin*dIII fragment from pUraGalU2.

U3 repression experiments

Yeast strains were cultured in a shaker at 30°C in YPD (2% glucose) or YPGal (2% galactose) (Sherman *et al.*, 1986). For the studies of U3 repression, freshly grown galactose cultures were either washed once in glucose medium and used to inoculate fresh pre-warmed glucose medium, or diluted directly into fresh pre-warmed galactose medium as controls, to give an optical density of ~0.5 at the zero time-point. The cultures were subsequently diluted to maintain exponential growth.

Extraction of RNA

Total RNA was extracted from yeast cells essentially as described by Rubin (1975): cells were harvested by centrifugation at 4°C, resuspended in 1 ml of cold water, collected by centrifugation in a micro-centrifuge for 2 s, then placed on ice. As soon as possible, the cells were resuspended in 0.5 ml 10 mM Tris−HCl, pH 7.5, 10 mM EDTA, 0.5% SDS and immediately mixed with 0.5 ml phenol (equilibrated with 10 mM Tris−HCl, pH 7.5, 1 mM EDTA, 0.2% 2-mercaptoethanol, 0.1% 8-hydroxyquinoline) at 70°C, vortexed vigorously, incubated for 30 min at 70°C with occasional vortexing, then placed on ice. The aqueous phase was extracted with 25:24:1 phenol/chloroform/isoamylalcohol and with 24:1 chloroform/isoamylalcohol. RNA was precipitated with ethanol and redissolved in water. The RNA concentration was estimated from the absorption at 260 nm. The RNA yield per cell was somewhat variable, according to the density of the culture at harvest, but no significant differences were observed between repressed and control strains.

Northern blotting and hybridization

Yeast pre-rRNA was separated by electrophoresis in vertical, 1.2% agarose gels containing 6% formaldehyde and 10 mM sodium phosphate, pH 6.5; 2 μg RNA was loaded in each lane. Small RNAs were resolved in 6% polyacrylamide (19:1) gels containing 7 M urea and 90 mM Tris−borate, 2.5 mM EDTA, pH 8.3. RNA was applied to the gels in 50% formamide after heating at 95°C for 2 min and chilling on ice. The RNA was subsequently transferred electrophoretically either to Nytran (Schleicher & Schüll) or to Zetabind (CUNO, Inc.) membranes at 300 mA, overnight, in 25 mM sodium phosphate, pH 6.5, at 4° C, and then fixed to the dried filters by ultraviolet irradiation.

Hybridization with DNA probes to pre-rRNA spacers was at 42° C in 50% formamide, 5×SSC, 0.5% SDS, 50 mM Tris−HCl pH 7.5, 5 mM EDTA, 5×Denhardt's reagent and 50 μg/ml denatured herring sperm DNA. The filters were subsequently washed at 55°C three times for 20 min in 2×SSC, 0.2% SDS, then three times in 0.2×SSC, 0.02% SDS and exposed to X-ray film. Hybridization to the end-labelled oligodeoxynucleotide '49' (5′-TAGATTCAATTTCGGTTT-3′), complementary to U3A, was at 30°C in the same medium as above but without formamide; washing was in 5×SSC, 0.5% SDS at 42°C. Relative quantities of specific RNAs on Northern blots were determined by liquid scintillation counting after cutting out labelled bands from the filter.

Probes for the spacers of pre-rRNA were prepared as follows. The 5′ ETS probe consists of a 375 bp *Dde*I fragment, extending from −172 to +203 with respect to the pre-rRNA transcriptional initiation site, isolated from a rDNA clone from a genomic DNA library of the wild-type yeast strain ATCC 25657 (Hughes *et al.*, 1987). The ITS1 probe consists of a 383 bp *Alw*I fragment extending from the 5′ boundary of ITS1 to 23 bp within the 5.8S domain, isolated from plasmid pBR-D. The ITS2 probe consists of a 270 bp *Eco*RI−*Cla*I fragment containing the 3′ portion of the 5.8S domain and 194 bp of ITS2, isolated from pBR-A. Plasmids pBR-A and pBR-D, containing the rDNA *Eco*RI fragments A and D, were kindly provided by Dr Thomas D.Petes. Probes were prepared from purified restriction fragments by random priming (Feinberg and Vogelstein, 1983).

Primer extension analysis

Primer extension reactions were performed with 2 μg total yeast RNA and a molar excess of primer essentially as described (Ares and Igel, 1990) except that annealing was for 45 min at 65°C following 2 min at 95°C, actinomycin D was omitted and the reactions were stopped by the addition of 200 μl 50 mM NaOH, 2 mM EDTA, boiled for 2 min, then precipitated by the addition of 25 μl 3 M sodium acetate, pH 5.2, and 0.5 ml ethanol. The oligonucleotides use for the experiment of Figure 5A were J6: 5′-ACTATCTTAAAACAAGAAGCAACAAGCAG-3′, complementary to the 5′ ETS, and J8: 5′-CATGCATGGCTTAATCTTTGAGACAAGC-3′, complementary to 18S rRNA.

Ribonuclease protection analysis

Ribonuclease protection analysis was performed as described (Melton, 1984) using an RNA probe complementary to 243 nt of pre-rRNA, from −181 to +62 relative to the 5′ end of mature 18S rRNA, transcribed *in vitro* using bacteriophage T7 RNA polymerase and [α-^{32}P]UTP from a cloned fragment of rDNA, linearized prior to transcription by cleavage at the *Hin*dIII site within the 5′ ETS. For each sample, 2 μg of total yeast RNA was annealed to the probe and digested with 1 μg/ml ribonuclease T1, or 40 μg/ml pancreatic ribonuclease A, at 30°C for 1 h.

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